## REMARKS/ARGUMENTS

Upon entry of the present amendment, claims 1-5, 17-19, 21-22, 24, 26, 29, 39, 41, 53, 55, 64, 88-90, and 92-100 are pending in the application, with claims 1-5, 17-19, 21-22, 24, 26, 29, 39, 41, 64, and 94-98 having been withdrawn from consideration for being directed to non-elected subject matter.

Claims 53, 55, 88-90, and 92 are amended, claims 54 and 91 are canceled, and new claims 99 and 100 are added in the present amendment. Support for the amendment to claim 53 can be found in the specification at, *e.g.*, paragraphs 0158 of the published application (US 2006/0258656 A1) and in original claim 55. Support for the amendment to claim 88 can be found in the specification at, *e.g.*, paragraphs 0171-0176 of the published application. Support for the amendment to claim 89 can be found in the specification at, *e.g.*, paragraph 0158 of the published application. Support for the amendment to claim 92 can be found in the specification at, *e.g.*, paragraphs 0160 and 0174 of the published application. Claim 55 is amended to reflect the amendment to claim 53 and the cancelation of claim 54. Claim 90 is amended to correct a grammatical error. Support for new claim 99 can be found in the specification at, *e.g.*, paragraphs 0160 and 0174 of the published application. Support for new claim 100 can be found in the specification at, *e.g.*, Example 21, including paragraph 0307 of the published application. No new matter is added by the present amendment.

Applicants address each of the Examiner's rejections and objections below in the order presented in the Office Action.

### *Claim Rejections under 35 U.S.C. 112, Second Paragraph*

The Examiner has rejected claims 53-55 and 88-93 under 35 U.S.C. 112, second paragraph. The Examiner stated that the terms "anti-neoplastic agent," "hypoxic activator," and "alkylating agent" are indefinite. The Examiner suggested that this rejection could be overcome by amending the claims to recite a structurally defined hypoxic activator and specific anti-neoplastic agents. *See* p. 4, first paragraph of Office Action.

Without agreeing with the Examiner's position, applicants have amended independent claim 53 to recite the specific structurally-defined hypoxic activator formerly recited in dependent claim 55. Applicants have also amended independent claims 53 and 89 to indicate that the anti-neoplastic agent is an alkylating agent, and submit that, as discussed below, the term "alkylating agent" has a clear meaning and does not render the claims indefinite.

The Examiner stated that the term "alkylating agent" is unclear. *See* p. 4, 1st paragraph of the Office Action. The claims, as amended, recite the term "alkylating agent" in the context of anti-neoplastic agents, *i.e.*, the anti-neoplastic agent is an alkylating agent. Applicants submit that "alkylating agent," as used in the claims with reference to an anti-neoplastic agent, is a well known term of art that would be immediately recognized by a skilled artisan as conveying a specific meaning. *See*, *e.g.*, Cancer, Principles and Practice of Oncology, 6th Edition, DeVita *et al.*, Lippencott Williams and Wilkins, Philadelphia, PA, pp. 363-376, 2001 (a copy of pages 363-376 are enclosed). DeVita *et al.* reports that antitumor alkylating agents react with DNA bases in cells to prevent cell replication. *See* p. 363. DeVita *et al.* also describes several classes of such alkylating agents, including agents such as cyclophosphamide, ifosfamide, melphalan, chlorambucil, and thiotepa (*see* pp. 363-366), which are also exemplified in the instant specification (*see* paragraph 0174 of the published application). The requirements of 35 U.S.C. 112, second paragraph, are satisfied if a person skilled in the field of the invention would reasonably understand the claim when read in the context of the specification. Here, the skilled person would understand what is meant by the term "alkylating agent," as used in the context of anti-neoplastic agents in both the specification and claims. Thus, use of the term "alkylating agent" does not render the claims indefinite.

The Examiner also stated that the breadth of the term "alkylating agent" prevents one from ascertaining the scope of the claims. *See* p. 4, first paragraph of the Office Action. However, breadth is not to be equated with indefiniteness. *See* MPEP §2173.04. Indefiniteness implies that one of skill would not be able to determine whether any particular anti-neoplastic agent is encompassed within the term "alkylating agent." As discussed above, the art and the specification make clear that an anti-neoplastic alkylating agent is an agent that reacts with DNA bases in cells to prevent cell replication. One of skill could readily determine that an alkylating

agent reacts with DNA bases in cells to prevent cell replication, and would be encompassed by the claims. Where the scope of the subject matter embraced by the claims is clear, then the claims comply with 35 U.S.C. 112, second paragraph. *See* MPEP §2173.04. Here, the scope of the claimed subject matter is clear because the term "alkylating agent," as used to define the anti-neoplastic agents of the claimed invention, is a term of art whose meaning would be understood by the skilled artisan. Thus, the full scope of the claimed invention is clear and the claims are not indefinite.

Based on the arguments presented above, applicants respectfully request withdrawal of this ground of rejection.

***Claim Rejection under 35 U.S.C. 112, First Paragraph***

The Examiner has rejected claim 88 under 35 U.S.C. 112, first paragraph, because the specification allegedly fails to enable the full scope of the claim. The Examiner suggested that this rejection could be overcome by amending the claim to recite specific cancers identified in the specification. *See* p. 9, second full paragraph of the Office Action.

Without agreeing with the Examiner's position, applicants have amended claim 88 to recite specific cancers identified in the application. Based on applicants' disclosure, the skilled artisan could practice the full scope of the claimed invention without undue experimentation.

Based on the arguments presented above, applicants respectfully request withdrawal of this ground of rejection.

***Claim Objections***

The Examiner has objected to claims 53-55, and 88-93 as encompassing non-elected subject matter. The Examiner suggested that applicants amend the claims to the scope of elected subject matter set forth at pages 2-3 of the Office Action. *See* p. 10, first paragraph of the Office Action.

Without agreeing with the Examiner's objections, applicants submit that the current claim amendments discussed above fully address the Examiner's objections.

Applicants wish to point out that the Examiner's claim objections appear to include an objection to the recitation of various classes of anti-neoplastic agents, including "alkylating agents," previously set forth in claim 89. Applicants have not been able to identify any such restriction requirement in the Office Action of May 5, 2008, which set forth a restriction of the claims to the presently claimed hypoxic activator, and required election of a single compound for examination. *See* pp. 4-5 of the Office Action of May 5, 2008. Thus, applicants submit that the current claim amendments fully address the Examiner's objections to the inclusion of non-elected subject matter.

Based on the arguments presented above, applicants respectfully request withdrawal of the Examiner's claim objections.

If the Examiner believes a telephone conference would expedite prosecution of this application, please telephone the undersigned at 650-326-2400.

Respectfully submitted,

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# CANCER

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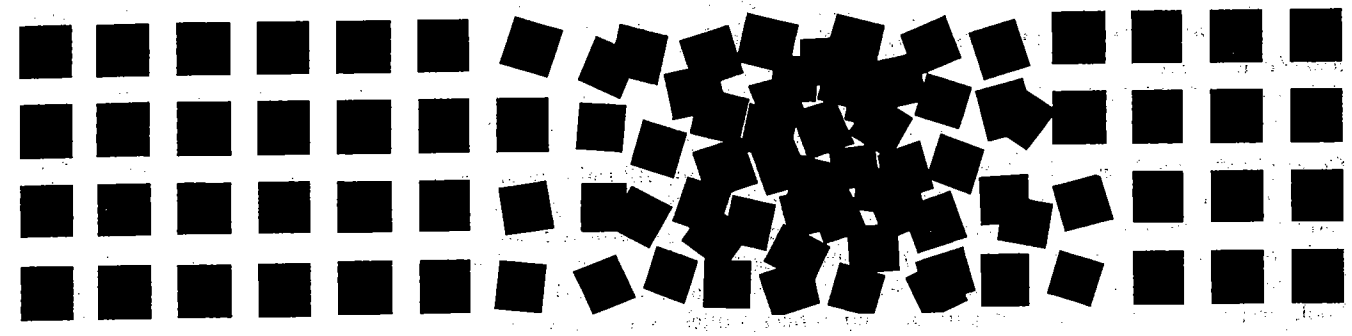

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SECTION 3

OLIVER MICHAEL COLVIN

# Antitumor Alkylating Agents

## HISTORY OF THE ALKYLATING AGENTS

A nitrogen mustard alkylating agent was the first nonhormonal chemical that demonstrated significant clinical antitumor activity. The clinical evaluation of nitrogen mustards as antitumor agents evolved from the observed clinical effects of sulfur mustard gas used as a weapon in World War I. This gas was used because of its vesicant effect on the skin and mucous membranes, especially the eyes and respiratory tract.[1] However, in addition to this deadly effect, depression of the hematopoietic and lymphoid systems was observed in victims and experimental animals.[2] These observations led to further studies that used the less volatile nitrogen mustards (Fig. 19.3-1). Studies published in 1946 demonstrated regression of tumors, especially lymphomas[3–5] and led to the introduction of the compound nitrogen mustard (mechlorethamine, Mustargen) into clinical practice. Subsequently, less toxic and more clinically effective nitrogen mustard derivatives and other types of alkylating agents have been developed.

## CHEMISTRY AND CYTOTOXICITY OF ALKYLATING AGENTS

The alkylating agents react with (or "alkylate") many electron-rich atoms in cells to form covalent bonds. The most important reactions with regard to their antitumor activities are reactions with DNA bases. Some alkylating agents are monofunctional and react with only one strand of DNA. Others are bifunctional and react with an atom on each of the two strands of DNA to produce a "cross-link" that covalently links the two strands of the DNA double helix. Unless repaired, this lesion will prevent the cell from replicating effectively. The lethality of the monofunctional alkylating agents results from the recognition of the DNA lesion by the cell and the response of the cell to that lesion. Analogous cellular reactions may occur to the interstrand cross-links, but such reactions have not been definitively established.

## CLASSES OF ALKYLATING AGENTS AND THEIR PROPERTIES

### NITROGEN MUSTARDS

#### *Mustargen*

Mustargen is currently used in the MOPP [Mustargen, vincristine (Oncovin), procarbazine, prednisone] regimen for the treatment of Hodgkin's disease[6] but rarely for other purposes. The other nitrogen mustards in significant clinical use are cyclophosphamide, ifosfamide, melphalan, and chlorambucil (Fig. 19.3-2). All these compounds produce cytotoxicity by forming covalent interstrand cross-links in DNA (as shown in Fig. 19.3-3 for Mustargen). The nitrogen mustard cross-link has been demonstrated to occur in the G-X-C/C-Y-G configuration,[7] as opposed to the G-C/C-G cross-link that had previously been predicted.[8] The formation of the G-X-C/C-X-G cross-link has been postulated to occur on the basis of the greater frequency of approximation of the N7 atoms of the two guanylates in the G-X-C/C-X-G configuration, as opposed to the G-C/C-G configuration.[9]

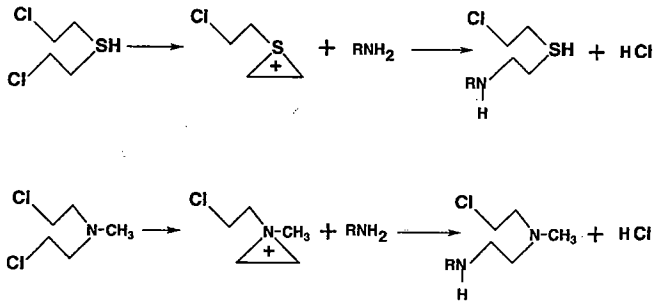


**FIGURE 19.3-1.** Structures and alkylation mechanisms for sulfur mustard and the nitrogen mustard, Mustargen (mechlorethamine).

Mustargen is available only as an intravenous preparation that can also be used topically for cutaneous malignancies. In the MOPP regimen, Mustargen is used at a dose of 6 mg/m$^2$ on days 1 and 8 of the monthly schedule. Toxicities unique to the agent are topical irritation and pain on injection if given too rapidly. The clearance of the drug is very rapid, but pharmacokinetics have not been performed with modern techniques.

#### *Cyclophosphamide*

The most frequently used alkylating agent, cyclophosphamide, is used for the treatment of breast cancer in combination with doxorubicin (Adriamycin)[10] or with methotrexate and 5-fluorouracil[11] and for the treatment of lymphomas,[12,13] childhood tumors,[14,15] and many solid tumors.[16] High doses of cyclophosphamide are frequently used in conjunction with bone marrow transplantation[17–19] and for the treatment of autoimmune diseases.[20,21]

Cyclophosphamide is inactive *in vitro* and is metabolized by P-450 enzymes in the liver to active species, as shown in Figure 19.3-4. The initial product is 4-hydroxycyclophosphamide (4-HC), which is released from the liver into the circulation.[22] This compound is in equilibrium with an open-ring tautomer, aldophosphamide. Aldophosphamide spontaneously eliminates acrolein to produce phosphoramide mustard,[23] which is an active bifunctional alkylating species.[24] Phosphora-

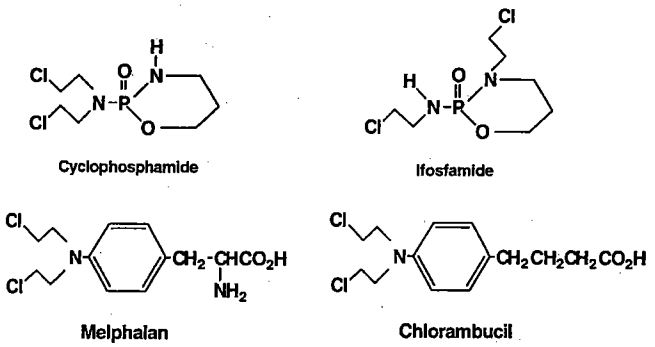



**FIGURE 19.3-2.** Nitrogen mustards in frequent clinical use.

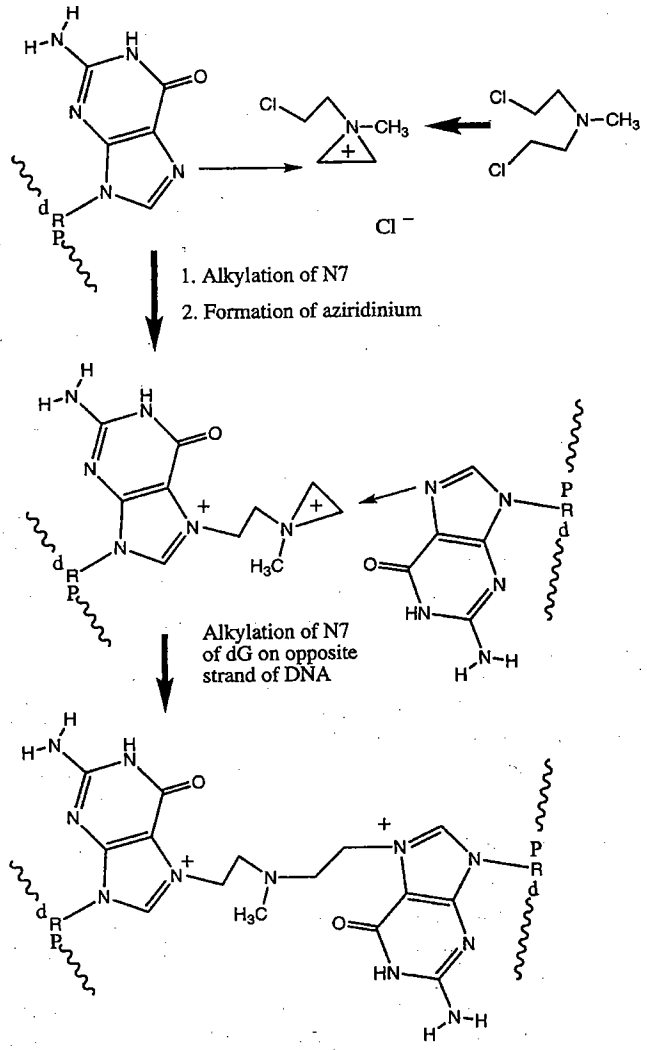



**FIGURE 19.3-3.** Alkylation of DNA and formation of interstrand cross-link by nitrogen mustard.

mide mustard is zwitterionic at physiologic pH[25] and enters cells poorly. 4-HC-aldophosphamide is not charged and enters cells facilely. While phosphoramide mustard is toxic to cells *in vitro* at concentrations of 100 μM and higher, 4-HC is cytotoxic in the range of 10 μM.[26] Thus, 4-HC-aldophosphamide serves as an efficient delivery system for phosphoramide mustard, which has been demonstrated to produce an interstrand DNA cross-link analogous to the cross-link produced by mechlorethamine.[7] Recent studies by Shulman-Roskes et al.[27] have demonstrated that phosphoramide mustard readily eliminates chloroethylaziridine,[27] which probably also plays a role in the cross-linking of DNA in cells exposed to 4-HC.

As shown in Figure 19.3-4, 4-HC is a substrate for the enzyme aldehyde dehydrogenase.[28] In cells that contain this enzyme, the bulk of the 4-HC is oxidized to carboxyphosphamide, which is not an active alkylating agent. Consequently, cells with high aldehyde dehydrogenase (ALDH) content are resistant to the metabolites of cyclophosphamide.[29,30] Early hematopoietic stem cells and megakaryocytes contain high levels, as do the epithelial stem cells in the small intestine and mucous membranes.[30,31] These observations explain why cyclophosphamide administration produces a shorter period of hematopoietic depression,[32] is relatively sparing of platelets, and is associated with less gastrointestinal toxicity and mucositis than other alkylating agents.[33]

4-HC is too unstable to be used as a reagent, but the compound 4-hydroperoxycyclophosphamide (see Fig. 19.3-4) is spontaneously converted in aqueous solution to 4-HC and can be used for *in vitro* studies of cell sensitivity.[34,35] This compound has also been used for the *in vitro* treatment of autologous bone marrow to reduce the number of tumor cells returned to the patient.[36]

Cyclophosphamide is available as tablets for oral administration or as an intravenous preparation. The drug is used at a variety of doses and schedules. Oral administration is particularly used for autoimmune diseases at a daily dose of approximately 100 mg. Because of its rapid absorption and high bioavailability, even very high doses can be given orally, but high intermittent doses are usually given intravenously. In moderate-dose combination chemotherapy, doses of cyclophosphamide in the range of 750 mg are usually used. For high-dose therapy in conjunction with hematopoietic cell transplantation, doses of up to 50 mg/kg for 2 or 4 days in combination with other agents are used.

The bulk (nearly 70%) of a dose of cyclophosphamide is excreted in the urine as the inactive carboxyphosphamide.[37,38] At high doses (approximately 50 mg/kg), plasma concentrations of up to 400 μM of cyclophosphamide are achieved,[38] and clearance depends on the renal clearance and the rate of microsomal metabolism in the liver. With improved and more facile techniques to measure 4-HC concentrations accurately, the clinical pharmacology of cyclophosphamide and this critical transport intermediate are being more carefully defined. Studies in patients receiving high-dose therapy have demonstrated considerable variation in the rates of clearance of cyclophosphamide between patients, with consequent differences in the peak concentrations (1 to 15 μM) and total exposure of the patient to 4-HC (60 to 140 μM.hours).[39,40] The total exposure to 4-HC is probably the major determinant of therapeutic effect. Currently, several programs are evaluating dose adjustment regimens based on the initial pharmacokinetics of cyclophosphamide and 4-HC. While it is known that substantial concentrations of phosphoramide mustard are present in plasma (up to 10 μM after 60 mg/kg of cyclophosphamide[38]), this concentration is well below the concentrations needed for *in vitro* cytotoxicity of phosphoramide mustard.[26]

A unique toxicity of cyclophosphamide and other oxazophosphorines is a characteristic hemorrhagic cystitis[41,42] due to irritation of the bladder mucosa from urinary metabolites. Acrolein has been identified as the metabolite most responsible for this effect,[43] but phosphoramide mustard and chloracetaldehyde may contribute to this toxicity. Careful hydration and emptying of the bladder are crucial to avoiding this toxicity, which has produced massive and even fatal hemorrhage. Another toxicity that has been associated with cyclophosphamide is an antidiuretic effect, especially at high doses.[44] This effect may produce marked fluid retention and electrolyte abnormalities, particularly low sodium, and seizures and fatalities have been seen.[45] It is important to avoid low-sodium-containing fluids after high-dose cyclophosphamide, and the fluid retention syndrome has been treated with furosemide to promote free water clearance.[46] The most severe dose-limiting toxicity of cyclophosphamide is a fulminant cardiac toxicity,[47] which is often fatal when seen clini-

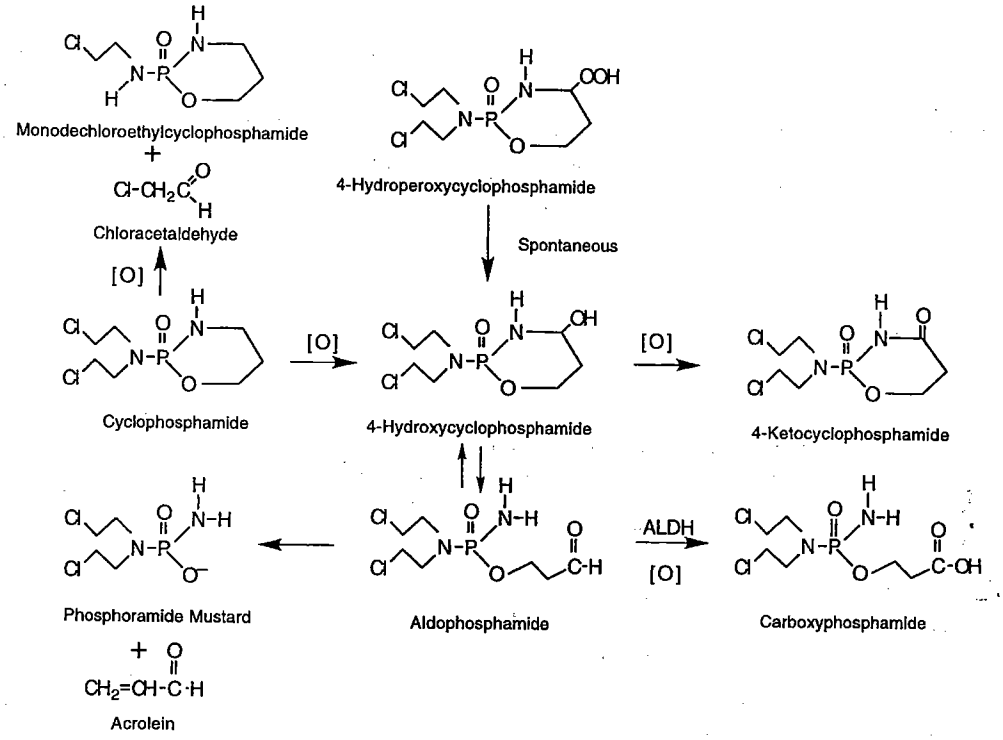



**FIGURE 19.3-4.** Metabolism of cyclophosphamide.

cally. This toxicity is seen only after the high doses used in bone marrow transplantation. It was initially seen in patients receiving 60 mg/kg/d of cyclophosphamide for 4 days, and the incidence has decreased since lower doses have been used. The syndrome usually presents with severe cardiac failure, beginning approximately 10 days after drug administration, with a dilated heart and low electrocardiogram voltage. There is a characteristic pathologic picture of edema, interstitial hemorrhage, and cardiac necrosis.[47]

### *Ifosfamide*

Ifosfamide is a structural isomer of cyclophosphamide that is often used in the treatment of sarcomas and pediatric tumors (see Fig. 19.3-2). There is more chloroethyl side chain oxidation of ifosfamide (up to 50%) than of cyclophosphamide (<10%), and the degree of such metabolism is more variable than with cyclophosphamide.[48] Oxidation of the chloroethyl groups produces chloroacetaldehyde, which is probably responsible for the neurotoxicity[49] and renal toxicity[50] that have been seen with ifosfamide therapy. Since the oxidation of a chloroethyl side chain produces a much less toxic monofunctional agent, higher doses of ifosfamide than cyclophosphamide must be used clinically. The studies of the clinical pharmacology of ifosfamide have been more limited than those of cyclophosphamide but have demonstrated large intrapatient variability in the pharmacokinetics and metabolism of the agent during repeated administrations.[51,52]

### *Melphalan*

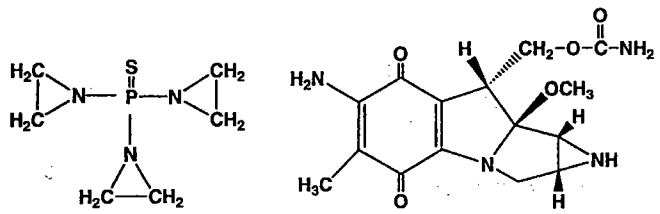
Melphalan is now used principally for the treatment of multiple myeloma,[53] for high-dose myeloablative therapy in conjunction with bone marrow transplantation,[54] and for the isolated limb perfusion of localized tumors,[55] especially malignant melanoma and sarcomas (see Fig. 19.3-2). Melphalan is an amino acid analogue and is actively transported into cells by amino acid transport systems.[56,57] It has been demonstrated that cellular uptake[58] and transport into the central nervous system (CNS)[59] of melphalan can be modulated by the amino acid content in the extracellular fluid.

Melphalan is available both as tablets and as an intravenous preparation. For the treatment of multiple myeloma, melphalan is usually used orally at a dose of 0.25 mg/kg for 4 days, with prednisone on the same schedule every 4 to 6 weeks. At these doses, peak plasma concentrations of 0.625 μM are found, but absorption is variable.[60] For bone marrow transplantation, doses of melphalan of 100 to 140 mg/m$^2$ are used.[61] At these doses, peak concentrations of melphalan of 40 to 50 μM are reached.[61,62]

### *Chlorambucil*

Chlorambucil is used for the treatment of B-cell chronic lymphocytic leukemia[63] and lymphomas[64] and for the immunosuppressive therapy of autoimmune diseases.[65] It is administered orally and is well tolerated when given either by daily administration or intermittent high-pulse doses.[64] Chlorambucil is well tolerated by most patients and can be used successfully for patients who have severe nausea and vomiting with cyclophosphamide or melphalan.

Chlorambucil is available only in an oral formulation. For chronic leukemia and immunosuppression, daily doses of 3 to 6 mg are given for a number of weeks, or 12 mg/m$^2$ may be given monthly. Pulsed dose pulse chlorambucil for lymphoma is given orally at a dose of 16 mg/m$^2$ daily for 5 consecutive days each month.[64] Chlorambucil is metabolized to a less active derivative—phenylacetic acid mustard—and the clinical pharmacology of chlorambucil is very similar to that of melphalan.[66]



Thiotepa Mitomycin C

**FIGURE 19.3-5.** Aziridine agents.

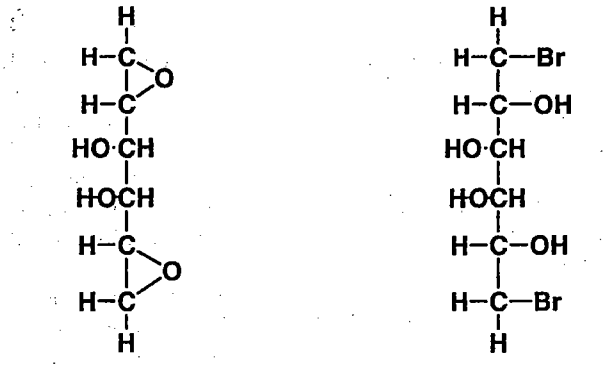
## AZIRIDINES AND EPOXIDES

The aziridine agents are related to the nitrogen mustards but contain uncharged aziridine rings that are less reactive than the aziridinium rings formed by most of the nitrogen mustards. The two aziridine agents that are frequently used clinically are thiotepa and mitomycin C (Fig. 19.3-5). The diepoxide dianhydrogalactitol reacts with DNA in a similar fashion to the aziridines but has been succeeded in clinical use by dibromodulcitol, which spontaneously generates dianhydrogalactitol *in situ* (Fig. 19.3-6).

### *Thiotepa*

Thiotepa is now used most frequently in combination with other alkylating agents in high-dose therapy with stem cell support.[39,67] Thiotepa has been demonstrated to react with the N7 position of guanylic acid in DNA[68] and to cross-link DNA,[69] indicating that it is acting similarly to the nitrogen mustards. Thiotepa is desulfurated by cytochrome P-450 enzymes[70] to produce tepa. Tepa is less toxic than thiotepa and has been demonstrated to produce alkali-labile sites in DNA, rather than cross-links.[69] These findings suggest that tepa reacts differently from thiotepa and produces monofunctional alkylation of DNA.

In combination with cyclophosphamide for high-dose therapy, thiotepa has been given as a continuous infusion for 4 days, at a daily dose of 200 mg/m$^2$. Under these conditions, steady-state levels of 2 to 6 μM of thiotepa are rapidly achieved.[71] Thiotepa is also used at a dose of 900 mg/m$^2$ in combination with high-dose cyclophosphamide and cisplatin.[72]



Dianhydrogalactitol Dibromodulcitol

**FIGURE 19.3-6.** Structures of dianhydrogalactitol and its prodrug, dibromodulcitol.

### *Mitomycin C*

Mitomycin C is an antibiotic extracted from a *Streptomyces* species and is used for the treatment of breast cancer,[73] esophageal cancer,[74] and gastrointestinal tumors.[75] As seen in Figure 19.3-5, this compound contains an aziridine ring. Particularly under hypoxic conditions, mitomycin C is reduced, with activation of the C1 position of the aziridine ring. This carbon then reacts in the minor groove with the extracyclic N2 amino group of a guanylic acid,[76,77] positioning the 10 carbon of the carbamate moiety to react with the N2 of a guanylic acid residue in an adjacent base pair in the complementary DNA strand. Mitomycin C and its reduced metabolites can also produce intrastrand guanylic acid–guanylic acid cross-links that produce bending of the DNA.[78]

In combination regimens, mitomycin C is given at doses of 10 to 15 mg/m$^2$ every 4 to 6 weeks. After a dose of 15 mg/m$^2$, peak plasma concentrations of 3 μM are seen.[79]

### *Dianhydrogalactitol*

Dianhydrogalactitol (see Fig. 19.3-6) is a hexitol derivative that contains two epoxide groups and cross-links DNA through the N7 atoms of guanylic acid,[80] presumably through the nucleophilic attack of the N7 atoms on the strained-ring epoxide groups. This compound was evaluated in clinical trials and demonstrated modest antitumor activity.[81,82] However, the structurally related dibromodulcitol (see Fig. 19.3-6) has demonstrated more antitumor activity[83,84] and is still being used in combination chemotherapy of breast cancer, cervical cancer, and brain tumors. Dibromodulcitol is hydrolyzed to dianhydrogalactitol, and its better antitumor activity is presumably due to more effective localization of the reactive agent in tumor cells.[85] Dibromodulcitol is usually administered at a dose of 1 g/m$^2$, which produces a maximum plasma concentration of approximately 50 μM.[86]

## ALKYL SULFONATES: BUSULFAN

Busulfan (Myeleran), other alkyl sulfonates, and the related sulfamates react with DNA by a direct displacement reaction (as shown in Fig. 19.3-7). Busulfan has been demonstrated to cross-link DNA,[87] but the structure of the cross-link has not been established. A chemically related agent, hepsulfam, with seven methylene units between the reactive groups, has been demonstrated to form a DNA G-X-C/C-X-G interstrand cross-link analogous to those formed by the nitrogen mustards.[88] Haddow and Timmis[89] reported in 1953 that busulfan was active against chronic myelogenous leukemia. Busulfan was for many years the principal agent used to treat this disease before being replaced by the use of hydroxyurea[90] and interferon-α,[91] both of which have proved to be more effective than busulfan. The most frequent use of busulfan in cancer therapy today is in high-dose therapy for many tumors, including chronic myelogenous leukemia, in conjunction with bone marrow or stem cell transplantation. For this application, high doses of busulfan are combined with cyclophosphamide, total body irradiation, or other agents.[18,92–94] The effectiveness of busulfan for this purpose is undoubtedly related to its marked myeloablative properties,[95] the mechanistic bases of which are not understood.

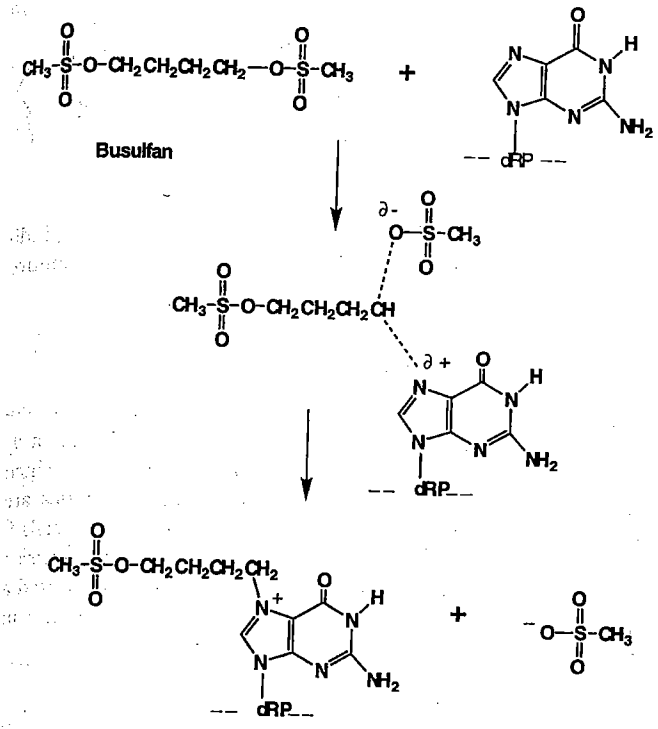



**FIGURE 19.3-7.** Alkylation of guanylate in DNA by busulfan through $S_N2$ alkylation. A second displacement reaction with the N7 of a guanylate in the complementary strand creates a G-X-C/C-X-G interstrand cross-link.

Until recently, busulfan was available only as an oral preparation, but intravenous preparations are now available. For hematopoietic transplantation, busulfan is usually given as 1 mg/kg every 6 hours for 4 days, for a total dose of 16 mg/kg. Peak concentrations of busulfan after each dose are approximately 10 μM.[96] High doses of busulfan have been associated with venoocclusive disease of the liver. This syndrome consists of hepatomegaly, jaundice, ascites, and hepatic failure with a high mortality rate.[97] Grochow et al.[96] have demonstrated that pharmacokinetic monitoring and dose adjustment of the busulfan can markedly reduce the incidence of venoocclusive disease.

## NITROSOUREAS

The members of the nitrosourea group of therapeutic alkylating agents are related to the alkylnitrosoamines and similar compounds that have long been known to be carcinogenic. Methylnitrosoguanidine and methylnitrosourea are monofunctional alkylating agents and were found to have modest antitumor activity.[98,99] Montgomery[100] and others[101,102] evaluated a number of analogues of these compounds and demonstrated remarkable antitumor effects of bischloroethylnitrosourea (BCNU; Fig. 19.3-8) against mouse tumors, and particularly against intracerebral tumors, which had been refractory to most agents because of the blood–brain barrier.[100–102] BCNU was found to produce interstrand cross-linking of DNA,[103] which has been demonstrated to occur through the spontaneous generation of a chloroethyldiazonium species[104] and the series of reactions illustrated in Figure 19.3-9.[105] As illustrated, this interstrand cross-link occurs between

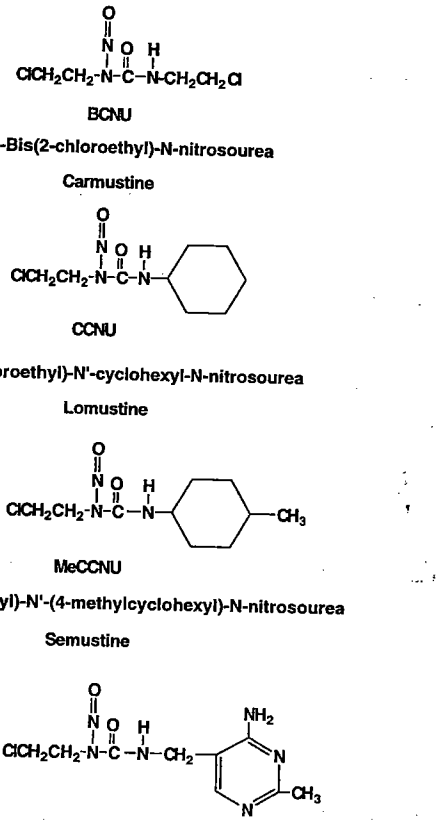



**FIGURE 19.3-8.** Nitrosoureas.

a guanylate in DNA and the base-paired cytidylate in the other strand of the DNA.[106]

### *Bischloroethylnitrosourea*

BCNU (carmustine; see Fig. 19.3-8) demonstrated activity against brain tumors clinically[107] and has continued to be used in the treatment of gliomas and other brain tumors. BCNU has also been used in the treatment of multiple myeloma[108] and in high-dose therapy in conjunction with bone marrow and stem cell transplantation.[109] BCNU can also be administered to brain tumors by direct injection[110] and by the implantation of biodegradable polymers containing BCNU into the brain.[111]

### *Cyclohexylchloroethylnitrosourea*

Cyclohexylchloroethylnitrosourea (CCNU, lomustine; see Fig. 19.3-8) is a more lipid-soluble nitrosourea. It is administered orally and is used in the treatment of brain tumors.[112,113]

### *Methylcyclohexylchloroethylnitrosourea*

Methylcyclohexylchloroethylnitrosourea (semustine; see Fig. 19.3-8) is an oral investigational drug that has been used in the treatment of gastrointestinal tumors.[114]

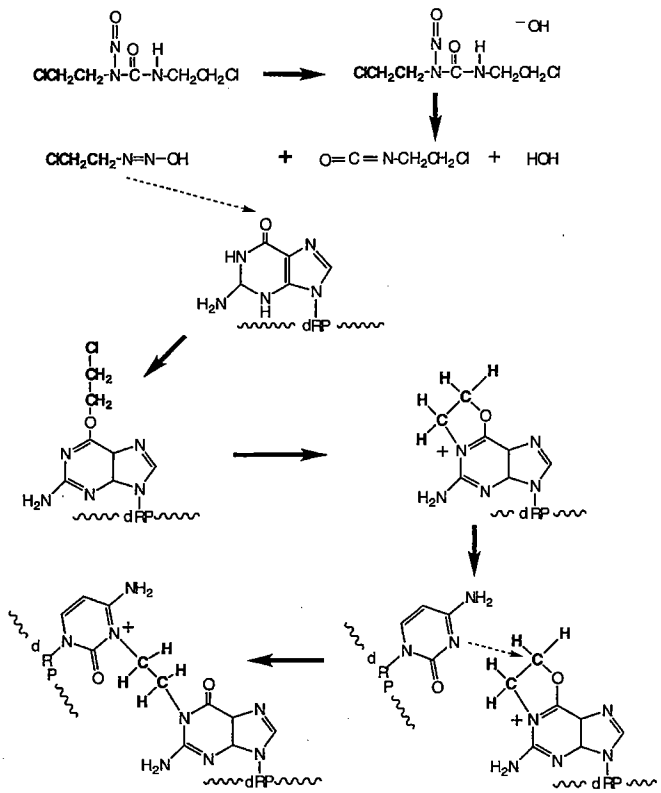


**FIGURE 19.3-9.** Reaction of BCNU with DNA to produce a G-C interstrand cross-link.

### *N'-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-N-(2-chloroethyl)-N-nitrosourea*

N'-[(4-amino-2-methyl-5-pyrimidinyl)methyl]-N-(2-chloroethyl)-N-nitrosourea (nimustine; see Fig. 19.3-8) is more water-soluble than the other chloroethylnitrosoureas and has been used for the treatment of CNS tumors by the intraarterial[115] and intrathecal routes.[116]

### *Clinical Pharmacology*

As a single agent, BCNU is usually used in a dose of 125 to 200 mg/m$^2$ every 6 to 8 weeks. In combination with doxorubicin for multiple myeloma, a dose of 30 mg/m$^2$ every 3 to 4 weeks has been used.[117] After doses in the range of 100 mg/m$^2$, peak plasma concentrations are in the range of 5 μM.[118] For high-dose therapy of breast cancer, BCNU is given at a dose of 600 mg/m$^2$ in combination with cyclophosphamide and cisplatin.[119] After this dose of BCNU, the peak plasma levels of BCNU have been shown to be approximately 5 μM.[120] Phenobarbital has been demonstrated to increase the clearance of BCNU[121] and to decrease the toxic and therapeutic effects. CCNU is administered in doses similar to those of BCNU. The parent CCNU has not been detected, but the peak concentrations of the ring hydroxylated metabolites are approximately 3 μM after doses of 130 mg/m$^2$.[122]

### *Specific Toxicities*

Hematopoietic toxicity of the nitrosoureas is severe and is delayed, with the nadir of the granulocytes occurring approximately 5 to 6 weeks after administration.[123] This finding indicates that these agents selectively damage a very primitive hematopoietic precursor.

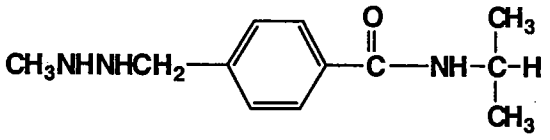


**FIGURE 19.3-10.** Procarbazine.

## HYDRAZINE AND TRIAZINE DERIVATIVES

The hydrazine and triazene derivative compounds are analogous to the nitrosoureas in that they decompose spontaneously or are metabolized to produce an alkyl carbonium ion, which alkylates DNA. Hydrazine and its substituted analogues are known carcinogens[124] that inhibit gluconeogenesis in cells[125] and have been promoted as antitumor agents.[126] However, objective preclinical and clinical studies have not supported a significant antitumor effect[127,128] for hydrazine analogues in general.

### *Procarbazine*

Procarbazine is a phenylhydrazine derivative that was initially developed as an inhibitor of monoamine oxidase but was found to have significant antitumor activity in preclinical models and clinically (Fig. 19.3-10).[129] Procarbazine was one of the components of the first effective combination chemotherapy regimen, MOPP, for Hodgkin's disease.[6] The agent is currently used for the treatment of Hodgkin's disease[6,130] and for the treatment of primary brain tumors.[113,131] Procarbazine has been demonstrated to be metabolized to a DNA-methylating agent,[132–134] which is most likely methylazoxyprocarbazine.[135,136] Since procarbazine is a monoamine oxidase inhibitor, patients can experience CNS depression[137] or stimulation[138] and acute hypertension, especially after the ingestion of tyramine-rich foods.

### *Dacarbazine*

Dacarbazine, or DTIC [(dimethyltriazeno)imidazole-carboxamide], is a triazene derivative that is metabolized by microsomal N-demethylation, predominantly in the liver, to an intermediate that spontaneously decomposes to release a methyldiazonium that methylates DNA (Fig. 19.3-11).[139–141] Dacarbazine is used in the regimen of doxorubicin, bleomycin, vinblastine, and dacarbazine for the treatment of Hodgkin's disease[130,142] and for the treatment of malignant melanoma.[119,143]

### *Temozolomide*

Temozolomide is a triazene analogue that spontaneously decomposes to produce a methyl diazonium ion, as illustrated in Figure 19.3-11.[144,145] This compound may produce a more homogeneous distribution of the short-lived MITC [(methyltriazeno)imidazole-carboxamide], which is spontaneously generated from temozolomide at all sites, than does dacarbazine, which is metabolized to MITC in the liver. The principal toxicities seen in phase I trials have been neutropenia and thrombocytopenia,

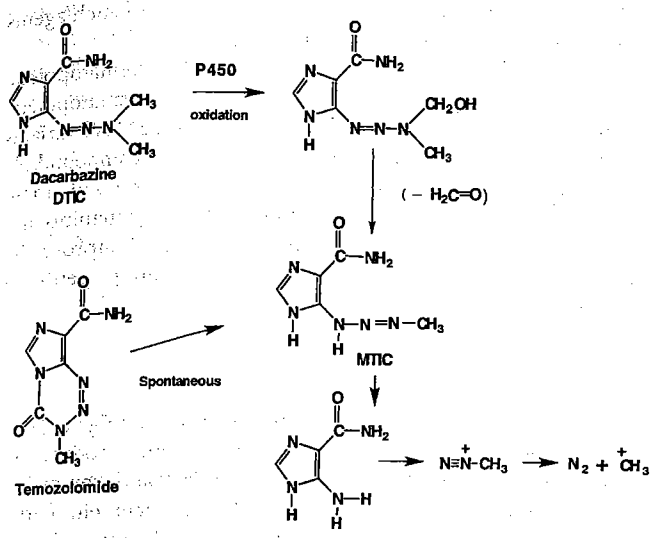



**FIGURE 19.3-11.** Generation of methyl diazonium from the triazenes dacarbazine and temozolomide.

and tumor responses were seen in those trials[146,147] in patients with glioma and melanoma. Phase II trials in patients with gliomas have shown response rates of 20% to 30%,[148,149] but phase II trials in patients with sarcomas[150] and pancreatic cancer[151] did not demonstrate significant responses.

These agents exert their toxicity predominantly through the methylation of the $O^6$ position of guanylic acid in DNA. Therefore, cells that contain significant $O^6$-alkyltransferase or are deficient in mismatch repair will be resistant to them (as discussed in the section Mechanisms of Toxicity and Drug Resistance).

Procarbazine is an oral preparation and used in the MOPP regimen for Hodgkin's disease at a dose of 100 mg/m$^2$/d for 14 days.[142] Because of its complex metabolism, pharmacokinetic studies have been limited. Dacarbazine is an intravenous preparation and is used in the regimen of doxorubicin, bleomycin, vinblastine, dacarbazine for Hodgkin's disease at a dose of 375 mg/m$^2$/d for 15 days.[142] For the treatment of malignant melanoma, a dose of 200 to 250 mg/m$^2$/d for 5 days is used and, at this dose, peak plasma concentrations of dacarbazine are approximately 30 μM.[152] This agent has been used as a single agent with bone marrow transplantation at a dose of 2000 mg/m$^2$.[153] At this dose, the maximum plasma concentration of dacarbazine was 800 μM.[153] Temozolomide is usually given orally at 150 to 250 mg/m$^2$/d for 5 days. Reid et al.[154] measured peak concentrations of MTIC of 0.5 to 5 μM after administration of these doses of temozolomide.[154] Baker et al.[155] studied the pharmacokinetics of $^{14}$C-labeled temozolomide and found peak concentrations of temozolomide of approximately 30 μM and peak concentrations of MTIC of approximately 1 μM.

## MECHANISMS OF TOXICITY AND DRUG RESISTANCE

### REACTION WITH CELLULAR MOLECULES

The alkylating agents are potent electrophiles and react with many electron-rich molecules within the cell to be inactivated.

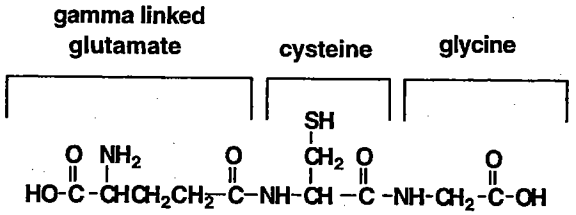



**FIGURE 19.3-12.** Structure of glutathione.

The principal such molecule is glutathione (GSH), a tripeptide with a free cysteine sulfhydryl that is present at millimolar concentrations in cells (Fig. 19.3-12). This small nucleophile is known to react with and inactivate virtually all the therapeutic alkylating agents, and a correlation between elevated cellular GSH concentrations and resistance to nitrogen mustards has been demonstrated.[156,157] The GSH S-transferase enzymes catalyze the conjugation of GSH with electrophiles, and increased activity of this class of enzymes enhances GSH-mediated resistance.[158–160] The GSH conjugates of specific alkylating agents have been characterized,[161–163] and the specific isoenzymes of GST that catalyze their formation have been characterized.[164–168]

Buthionine sulfoximine is an inhibitor of gamma-glutamylcysteine synthetase, the rate-limiting enzyme in the GSH synthesis pathway, and decreases the GSH concentration in cells.[169] Exposure to this compound sensitizes both normal and tumor cells to alkylating agents.[156,170,171] In a phase I clinical trial, buthionine sulfoxime has been shown to increase the hematologic toxicity of melphalan[172] and is currently in further clinical trials to determine whether this agent can increase the clinical antitumor efficacy of melphalan.

Cells can also be sensitized to alkylating agents by exposure to inhibitors of GSH S-transferases,[173,174] and a clinical trial of the GSH S-transferase inhibitor sulfasalazine with melphalan demonstrated increased nausea and vomiting but no increase in hematopoietic toxicity.[175] The membrane transporter multidrug resistance protein is known to mediate the efflux of GSH conjugates from the cell,[176] and Barnouin et al.[177] have demonstrated that this system can transport the GSH conjugates of chlorambucil and melphalan from cells. The observations suggest that modulation of these systems could enhance the efficacy of alkylating agents.

Kelley et al.[178] demonstrated that transfection of metallothionein into cells produced increased resistance to chlorambucil and melphalan. Subsequently, Yu et al.[179] have demonstrated that the thiol groups of metallothionein will bind melphalan and phosphoramide mustard.[180] It has also been demonstrated that exposure of cells to zinc will increase metallothionein concentration in the cell and increase resistance of the cells to melphalan, doxorubicin, and cisplatin.[181]

### ENHANCED DNA REPAIR: $O^6$ ALKYLATION

Another mechanism of cellular resistance to alkylating agents is repair of the DNA damage that the agents produce. The most defined mechanism of cellular repair of alkylating agent damage is that of the enzyme $O^6$-alkylguanine-alkyltransferase. As illustrated in Figure 19.3-13, this enzyme can remove an alkyl group from the $O^6$ position of guanine, and the alkylated enzyme is then rapidly degraded.[182] This mechanism has been shown to be effective in protecting normal and tumor cells

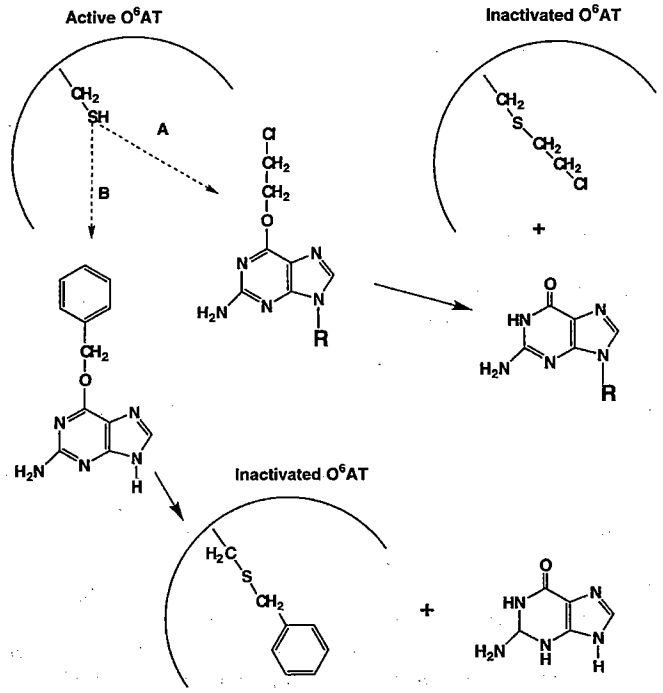



**FIGURE 19.3-13.** Interactions of $O^6$-alkylguanine-DNA alkyltransferase. Pathway A: Repair of $O^6$ alkylation by $O^6$AT. Pathway B: Inactivation of $O^6$AT by benzylguanine.

from the carcinogenic and toxic effects of DNA methylating agents, such as temozolomide and procarbazine.[183] Erickson et al.[184] demonstrated that this enzyme would also remove the 6-chloroethyl lesion produced by the alkylation of guanine by the chloroethylnitrosoureas and produce resistance to these compounds, and this observation has been confirmed and extended.[185]

It has been shown that such compounds as $O^6$-benzylguanine will be acted on by $O^6$-alkylguanine-DNA alkyltransferase (see Fig. 19.3-13) to remove the benzyl group[186] and that the enzyme will be rapidly degraded and depleted. Such compounds have been demonstrated to reverse tumor resistance due to $O^6$AT to the $O^6$ alkylating agents *in vitro* and *in vivo*,[187,188] and clinical trials of the combination of such agents and $O^6$-methylguanine are currently in progress.[189,190]

However, inhibitors of $O^6$AT enhance the hematopoietic toxicity of $O^6$ alkylating therapeutic agents. Hematopoietic stem cells have been successfully transfected with $O^6$AT variants that are resistant to $O^6$-benzylguanine and related compounds.[191] The hematopoietic systems of animals populated with these cells are resistant to the combination of $O^6$-benzylguanine and BCNU,[192] and clinical trials of this approach to improve the efficacy of chloroethylnitrosoureas and methylating agents are planned.

## CROSS-LINK REPAIR

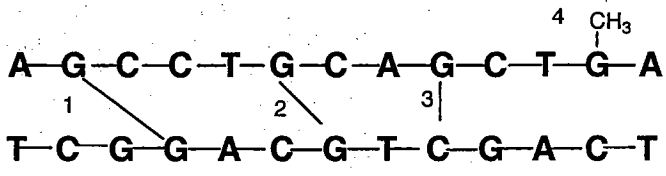
The use of alkaline elution and other techniques (Fig. 19.3-14) has demonstrated that DNA interstrand cross-links produced by nitrogen mustards can be removed in bacteria[193] and mammalian cells.[194] The mechanism of such repair has not been elucidated, but nucleotide excision repair[195] and poly(adenosine diphosphate–ribose) polymerase[196] appear to play a role.

Caffeine and related compounds have been demonstrated to enhance the cytotoxicity of nitrogen mustard.[197] This effect was associated with abrogation of $G_2$ arrest. O'Connor et al.[198,199] demonstrated that the $G_2$ arrest associated with nitrogen mustard resistance was associated with decreased activity of cdc2 kinase in the resistant cells. Caffeine has also been shown to inhibit nucleotide excision repair by binding to the subunit that recognizes the damage and helps to mediate this repair activity.[200] Elevated Bcl-2 has also been associated with nitrogen mustard resistance.[201]

A medulloblastoma cell line has been demonstrated to be resistant to activated cyclophosphamide (4-hydroperoxycyclophosphamide) on the basis of increased removal of DNA interstrand cross-links.[34,202] This cell does not appear to repair cross-links produced by BCNU and busulfan, indicating that the recognition of the nitrogen mustard cross-link is fairly specific.

## *IN VIVO* RESISTANCE

Kobayashi et al.[203] and St. Croix et al.[204] have described resistance to alkylating agents and other antitumor agents that is




| 1. G-X-C/C-X-G interstrand crosslink | 2. G-C/C-G interstrand crosslink | 3. G/C interstrand crosslink | 4. $0^6$-G Methylation |
|---|---|---|---|
| Nitrogen mustards<br>Thiotepa ?<br>Busulfan ? | Mitomycin C | Chloroethylnitrosoureas | Procarbazine<br>Temozolomide<br>Dacarbazine |
| Must be repaired for cell survival. Mustard crosslink can be excised by some resistant cells | Must be repaired for cell survival. Excision of crosslink not demonstrated. | Must be repaired for cell survival. Initial chloroethyl alkylation can be removed by O-6-AT to prevent crosslink. Excision of crosslink not demonstrated. | Recognition by mismatch repair necessary for toxicity. O-6 methyl can be removed by O-6AT. |

**FIGURE 19.3-14.** DNA lesions produced by alkylating agents.

associated with aggregation of tumor cells. This resistance is present when the tumor cells are growing *in vivo* or in three-dimensional *in vitro* culture with adherence between the cells but is not present when the cells are dispersed in two-dimensional culture. This type of resistance has also been associated with increased metastatic potential.[205]

## COMMON TOXICITIES

Toxicities that are associated with specific alkylating agents are described in the discussions of the individual agents. The toxicities common to the alkylating agents as a class are described here.

### HEMATOPOIETIC TOXICITY

The usual dose-limiting toxicity for an alkylating agent is hematopoietic toxicity. As described, cyclophosphamide usually produces a relatively rapid nadir of the granulocytes, with recovery within 3 weeks after a single dose or short course.[32,33,206] Cyclophosphamide is also relatively platelet-sparing. The reason for the relative hematopoietic sparing properties of cyclophosphamide is the high concentrations of the enzyme aldehyde dehydrogenase in hematopoietic stem cells and megakaryocytes.[30,31]

The nitrosoureas produce an unusual delayed hematopoietic toxicity, with nadirs of both granulocytes and platelets at 5 to 6 weeks after administration.[123] Severe granulocytopenia and thrombocytopenia are also characteristic of busulfan.[207] An interesting characteristic of busulfan is its relative sparing of lymphocytes. The different hematopoietic effects of alkylating agents, except for the characteristics of cyclophosphamide, are not explained but suggest significant differences in selectivity of the agents for hematopoietic precursors.

### GASTROINTESTINAL TOXICITY

The alkylating agents frequently produce nausea and vomiting, although this effect is usually not as severe as with the platinum agents. Cyclophosphamide produces severe nausea and vomiting in some patients, but these patients usually tolerate chlorambucil, which is clinically less emetogenic. The nausea and vomiting produced by alkylating agents are known to be mediated significantly through the CNS.[208,209] With the higher doses of alkylating agents used in bone marrow transplantation, increased nausea and vomiting are seen but can usually be controlled by corticosteroids and the newer antiserotonin antiemetics.[210–212] The alkylating agents can cause significant toxicity to the gastrointestinal mucosa and produce mucositis, stomatitis, and diarrhea, especially with the high doses of melphalan and thiotepa used in bone marrow transplantation.[213]

### GONADAL TOXICITY

The alkylating agents can produce significant gonadal toxicity. The characteristic testicular lesion in men is depletion of germ cells without damage to the Sertoli cells, which was first described with nitrogen mustard in 1948.[214] This lesion is also seen, often in association with oligospermia or aspermia, after treatment with other alkylating agents.[215,216] Spermatogenic dysfunction is reversible in some patients.[217,218]

Women treated with alkylating agents may develop amenorrhea associated with a marked decrease in ovarian follicles.[215,219,220] This complication and its irreversibility increase with the age of the woman.[221]

### PULMONARY TOXICITY

Interstitial pneumonitis and fibrosis were initially reported as a consequence of busulfan therapy[222] but have subsequently been reported to occur after therapy with melphalan,[223] chlorambucil,[224] cyclophosphamide,[225,226] mitomycin C,[227] and BCNU.[228,229] The clinical manifestations of this toxicity are dyspnea and a nonproductive cough, which can progress to cyanosis, pulmonary insufficiency, and death. The syndrome has particularly been associated in frequency and severity with high doses of BCNU.[230,231] The greater pulmonary toxicity of BCNU may be due to the spontaneous decomposition of BCNU, which produces chloroethyl isocyanate in addition to the alkylating chloroethyl diazonium moiety described.[232] Chloroethyl isocyanate is an analogue of methyl isocyanate, a known pulmonary toxin that produced many deaths when released in an industrial accident in Bhopal, India.[233]

### ALOPECIA

Alopecia from chemotherapy was first described after administration of dimethylmyeleran, an analogue of busulfan.[234] The alkylating agents now most associated with alopecia are cyclophosphamide and ifosfamide. Feil and Lamoureux[235] examined the alopecia-producing effects of metabolites and analogues of cyclophosphamide and proposed that the alopecic effect was due to the facile entry of a lipophilic metabolite (now known to be 4-HC) into the hair follicles. This hypothesis is consistent with the fact that vincristine, doxorubicin, and the taxanes, all associated with alopecia, are fairly lipophilic.

### TERATOGENICITY

All the therapeutically used alkylating agents are teratogenic in animal studies.[236–239] A review of the literature in 1968 found that 4 of 25 children born to mothers who received alkylating agents during the first trimester of pregnancy had fetal malformations.[240] On the basis of the limited information available, women treated with an alkylating agent during the first trimester of pregnancy may have a risk as high as 15% of having a malformed infant. Administration of alkylating agents during the second and third trimesters has not been associated with increased fetal malformations.[241,242] More recent reviews support the lack of malformations produced by treatment during the second and third trimesters,[243,244] and one review cites 19 women treated during the first trimester with no infant malformations.[244]

### CARCINOGENESIS

In the 1970s, there were reports of acute leukemia occurring in patients who had been treated with alkylating agents,[245–249] and subsequent experience has confirmed the occurrence of this complication. The incidence of leukemia is difficult to estimate because of the variety of agents, doses, and combinations used but is probably approximately 5%. In one group of 12 ovarian cancer patients receiving a high dose of melphalan, 4 devel-

oped acute leukemia.[248] In one report, the incidence of leukemia was found to be higher after melphalan treatment than after cyclophosphamide therapy.[250] This observation may be related to the stem cell–sparing properties of cyclophosphamide.[30] An increased frequency of solid tumors also occurs after alkylating agent therapy.[251,252]

## IMMUNOSUPPRESSION

In 1921, Hektoen and Corper[253] reported an inhibitory effect of sulfur mustard on antibody production. While all the alkylating agents produce some degree of immunosuppression, cyclophosphamide is the most immunosuppressive.[254] Cyclophosphamide and chlorambucil are the alkylating agents most commonly used for the treatment of autoimmune diseases.[255–259]

Selective inhibition of immunosuppressor cells with low doses of an activated analogue of cyclophosphamide and with melphalan has been demonstrated *in vitro*[260–263] and *in vivo*[263,264] and enhancement of the immune response has been shown *in vivo*.[263] For this reason, low doses of cyclophosphamide have been used in conjunction with immunotherapy.[265,266] Because of its potent immunosuppressive properties, cyclophosphamide has long been used in preparative regimens for allogeneic stem cell transplantation for malignancy[267] and more recently for the autologous transplantation of autoimmune disease.[268,269] The use of high doses of cyclophosphamide without stem cell support has now been reported to produce complete remissions in autoimmune diseases.[21,270,271]

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*SECTION* **4**

# *Cisplatin and Its Analogues*

The platinum drugs represent a unique and important class of antitumor compounds. Alone or in combination with other chemotherapeutic drugs, *cis*-diamminedichloroplatinum (II) (cisplatin) and its analogues have made a significant impact on the treatment of a variety of solid tumors. The realization that platinum complexes exhibit antitumor activity arose somewhat serendipitously in a series of experiments carried out by Rosenberg and colleagues beginning in 1961.[1] These studies involved determining the effect of electromagnetic radiation on the growth of bacteria in a chamber equipped with a set of platinum electrodes. Exposure of the bacteria to an electric field resulted in a profound change in their morphology and, in particular, the appearance of long filaments that were several hundred times longer than that of their untreated counterparts. This effect was not due to the electric field directly, but to the electrolysis products produced from the platinum electrodes. An analysis of these products revealed that the predominant species was ammonium chloroplatinate $[NH_4]_2[PtCl_6]$. This compound was inactive at reproducing the filamentous growth originally observed; however, Rosenberg and colleagues[1] soon discovered that the conversion of this complex to a neutral species by UV light resulted in an active species. Attempts to synthesize the active neutral platinum complex failed. They realized, however, that the neutral compound could exist in two isomeric forms, *cis* or *trans*, and that the latter species is the one they had synthesized. Subsequently, the *cis* isomer was synthesized and shown to be the active compound.

The observation that *cis*-diamminedichloroplatinum (II) and *cis*-diammine tetrachloroplatinum (IV) inhibited bacterial growth led to the testing of four neutral platinum compounds for antineoplastic activity in mice bearing the Sarcoma-180 solid tumor and L1210 leukemia cells.[2] All four compounds showed significant antitumor activity, with *cis*-diamminedichloroplatinum (II) exhibiting the most efficacy. Further studies in other tumor models confirmed these results and indicated that cisplatin exhibited a broad spectrum of activity. Although early clinical trials demonstrated significant activity against several tumor types, particularly testicular tumors, the severe renal and gastrointestinal toxicity caused by the drug nearly led to its abandonment. Cvitkovic et al.[3,4] showed that these effects could be ameliorated, in part, by aggressive prehydration, which rekindled interest in its clinical use. Currently, cisplatin is curative in testicular cancer and significantly prolongs survival in combination regimens for ovarian cancer. The drug also has therapeutic benefit in head and neck, bladder, and lung cancer.[5]

The unique activity and toxicity profile observed with cisplatin has fueled the development of platinum analogues that are less toxic and more effective against a variety of tumor types, including those that have developed resistance to cisplatin. Two other platinum drugs are widely used: *cis*-diammine-cyclobutanedicarboxylato platinum (II) (carboplatin) and 1,2-diaminocyclohexaneoxalato platinum (II) (oxaliplatin). Several new analogues with unique activities are currently in various stages of clinical development. Continued progress in the development of superior analogues requires a thorough understanding of the chemical, biological, pharmacokinetic, and pharmacodynamic properties of this important class of drugs. A review of these properties is the focus of this chapter.

## PLATINUM CHEMISTRY

Platinum exists primarily in either a 2+ or 4+ oxidation state. These oxidation states dictate the stereochemistry of the carrier ligands and leaving groups surrounding the platinum atom. Platinum (II) compounds exhibit a square planar geometry, whereas platinum (IV) compounds exhibit an octahedral geometry. Interconversion of the two oxidation states may readily occur. However, the kinetics of this reaction depend on the nature of the bound ligands. The nature of the ligands also determines the stability of the complex and the rate of substitution. For platinum (II) compounds, the rate of substitution of a ligand is strongly influenced by the type of ligand located opposite to it. Therefore, ligands that are bound more strongly stabilize the moieties that are situated *trans* to it. For *cis*-diamminedichloroplatinum (II), the two chloride ligands are prone to substitution, whereas substitution of the amino groups is thermodynamically unfavorable.[6] The stereochemistry of platinum complexes is critical to their antitumor activity, as evi-